SUPPORTING INFORMATION

Molecular Characterization of the Cercosporin Biosynthetic Pathway in the Fungal Plant Pathogen *Cercospora nicotianae*

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EXPERIMENTAL METHODS

Reagents and Biological Strains. All reagents and primers were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified. Magnesium chloride, sodium chloride, potassium phosphate monobasic, potassium phosphate dibasic, and deoxynucleotides (dNTPs) were purchased from ThermoFisher Scientific (Waltham, MA). Isopropyl-β-D-thiogalactoside (IPTG), kanamycin, and nickel (high density) agarose beads were purchased from Gold Biotechnology (St. Louis, MO). Imidazole was purchased from Acros Organics (Geel, Belgium). Difco™ potato dextrose agar (PDA) and Difco™ potato dextrose broth (PDB) were purchased from Beckton, Dickson, and Company (Franklin Lakes, NJ). Yeast extract and tryptone were purchased from Boston BioProducts (Ashland, MA). Agar was purchased from bioWORLD (Dublin, OH). Plasmids pET-24a(+) and pET-28a(+) were purchased from EMD Millipore (Darmstadt, Germany). All enzymes used for manipulation of DNA and 10 × HF Buffer were purchased from New England Biolabs (Ipswich, MA). Bio-Rad protein assay dye for determining protein concentration by the Bradford assay was purchased from Bio-Rad laboratories (Hercules, CA). Bacterial strain *Escherichia coli* BL21(DE3) was purchased from

Sigma-Aldrich and made electrocompetent by standard protocols. DNA purified from *C. nicotianae* (ATCC© 18366™) was provided by Dr. Kuang-Ren Chung (currently of National Chung Hsing University, Taichung, Taiwan). *C. nicotianae* (ATCC© 18366™) wild-type strain and cercosporin gene cluster knockout strains previously prepared were also generously provided by Dr. Chung.[1-5]

Culture Conditions. *C. nicotianae* (ATCC© 18366™) and knockout strains were maintained on Difco™ Potato Dextrose Agar (PDA). PDA plates were strictly maintained at a thickness of 3 mm. Starter cultures were prepared by spotting a colony from a stock plate on PDA with a sterile toothpick. The starter culture was allowed to grow, inverted, at 28 °C for 7 days under constant fluorescent light. Stock plates were prepared from fresh starter cultures that were wrapped in parafilm and stored at 4 °C in the dark until further use. Production cultures were prepared by spotting colonies from a fresh starter culture on PDA with a sterile toothpick. Colonies were spotted in a 2 cm grid. Mycelia were incubated, inverted, under constant fluorescent light at 28 °C for 7 days.

Metabolite Extraction and Purification. The total mass (mycelia and agar) from production cultures were collected, frozen in liquid nitrogen, and ground to a powder in a mortar and pestle. The powder was lyophilized for 12–16 h then suspended in water acidified with concentrated HCl. Metabolites were extracted from this slurry thrice with ethyl acetate at 4 °C. The ethyl acetate fractions were combined, washed with brine, dried with sodium sulfate, and evaporated under vacuum. The solid residue was stored at 4 °C until further use. Extracts were further purified by Sephadex LH-20 (GE Healthcare, Little Chalfont, UK) column chromatography and HPLC, as described below.

The extracted metabolites for *C. nicotianae* cultures were dissolved in methanol and filtered through 0.4 μm PTFE filters. Metabolites were separated on a Sephadex LH-20 column (GE Healthcare, Little Chalfont, UK) using methanol as a mobile phase with a flow rate of 1.3 mL/min and injecting 1.2 mL aliquots. Fractions were collected every 5 min. Fractions were screened for metabolites of interest by reverse phase HPLC on an Agilent 1200 (Agilent Technologies, Santa Clara, CA). Solvent A was water + 0.1% formic acid. Solvent B was acetonitrile + 0.1% formic acid. Fractions were injected onto a linear gradient of 5–95% solvent B over 10.8 min at 1.25 mL/min on a Kinetex XB-C18 column (4.6 mm × 75 mm, 2.6 μm, Phenomenex, Torrence, CA). Typical injection volumes were 10–15 μL. Chromatograms at 250 and 280 nm were recorded.

Fractions containing metabolites of interest were combined, concentrated under vacuum, and purified by reverse phase HPLC on an Agilent model 1100 (Agilent Technologies). Solvent A was water + 0.1% formic acid. Solvent B was acetonitrile + 0.1% formic acid. Fractions were injected onto a linear gradient of 5–95% solvent B over 45 min at 4.726 mL/min on a Prodigy 5u ODS3 column (10 mm × 250 mm, 5 μm, Phenomenex). Injection volumes were typically 500–1000 μL. Chromatograms at the expected λ_{max} for the metabolite of interest were recorded. Metabolites were manually collected as they eluted and the fractions from serial injections were combined. Acetonitrile and formic acid were removed from these samples by evaporation under vacuum in a rotary evaporator. The remaining aqueous solution was frozen in liquid nitrogen and lyophilized until dry. Purified samples were stored at 4 °C until further use.

Complementation Assays. Cercosporin biosynthesis functional knockouts were plated adjacent to one another to assay for cercosporin production by complementation. Colonies were spotted with a sterile toothpick from a fresh starter culture on 3 mm thick PDA plates. The

complete set of pairwise combinations of cercosporin biosynthetic functional knockouts was assayed. Colonies from different strains were spotted 0.5, 0.7, 1.0, 1.5, and 2.0 cm apart from one another. The cultures were incubated, inverted, under constant fluorescent light at 28 °C for 7 days. The appearance of red pigmentation at the boundary between two mycelia was taken to represent cercosporin production complementation. Evidence of complementation was monitored daily.

Preparation of Expression Constructs. DNA manipulations were carried out in accordance with established procedures.[6] Protocols for PCR and overlap extension PCR are described below. Details of the expression plasmids used in this study are summarized in Table S2. Expression constructs for CTB2, CTB3, and monodomain fragments of CTB3—the *N*-terminal methyltransferase (CTB3-MT) and the *C*-terminal monooxygenase (CTB3-MO)—were prepared with 6 × His tags for ease of purification by nickel affinity chromatography. Cut sites for protein deconstruction were guided by a variety of bioinformatics analyses including multiple sequence alignment, secondary structure prediction and the UMA algorithm for predicting interdomain regions.[7] Primer sequences used for cloning new constructs used in this study are presented in Table S1. All expression plasmids were maintained in *E. coli* BL21(DE3) cells stored in 20% glycerol at –80 °C.

The complete CTB3 ORF was cloned into a pCR®-Blunt vector (ThermoFisher Scientific) for easier genetic manipulation. The insert was prepared by PCR using *C. nicotianae* (ATCC© 18366™) genomic DNA template with primers CTB3-5 and CTB3-3. The product was ligated into pCR®-Blunt using the Zero Blunt® PCR Cloning Kit (ThermoFisher Scientific) according to the manufacturer's instructions, yielding pCR-CTB3.

Two full-length CTB3 expression constructs were prepared. The first had a *C*-terminal 6 × His tag (pECTB3) and the second had an *N*-terminal 6 × His tag (p28CTB3). The CTB3 ORF contains three exons that were synthetically spliced using overlap extension PCR. Overlap extension PCR DNA fragments were prepared by PCR from pCR-CTB3 template. The exon 1 fragment was prepared with primers CTB3-AseI-5 and CTB3-ex1-3. The exon 2 fragment was prepared with primers CTB3-ex2-5 and CTB3-ex2-3. Two exon 3 fragments were prepared with primers CTB3-ex3-5 and CTB3-NotI-3 or CTB3-Stop-3, for fragments without and with a stop codon, respectively. The resulting PCR products were spliced together using overlap extension PCR with outside primers CTB3-AseI-5 and CTB3-NotI-3 or CTB3-Stop-3 for inserts without and with a stop codon, respectively. The inserts were digested with AseI and NotI-HF. The insert without a stop codon was ligated in NdeI and NotI-HF digested pET-24a(+) yielding pECTB3. The insert with a stop codon was ligated in NdeI and NotI-HF digested pET-28a(+) yielding p28CTB3.

Two CTB3 *O*-methyltransferase monodomain expression constructs were prepared by PCR. The first had a *C*-terminal 6 × His tag (pECTB3-MT1) and the second had an *N*-terminal 6 × His tag (p28CTB3-MT1). The inserts were prepared by PCR using pECTB3 template with primers CTB3-AseI-5 and CTB3-MT1-3 or CTB3-MT1-Stop-3 for inserts without or with a stop codon, respectively. The inserts were digested with AseI and NotI-HF. The insert without a stop codon was ligated into NdeI and NotI-HF digested pET-24a(+) yielding pECTB3-MT1. The insert with a stop codon was ligated into NdeI and NotI-HF digested pET-28a(+) yielding p28CTB3-MT1.

Two CTB3 flavin-dependent monooxygenase monodomain expression constructs were prepared by PCR. The first had a *C*-terminal 6 × His tag (pECTB3-MO3) and the second had an

N-terminal 6 × His tag (p28CTB3-MO3). The inserts were prepared by PCR using pECTB3 template with primers CTB3-MO3-5 and CTB3-NotI-3 or CTB3-Stop-3 for inserts without or with a stop codon, respectively. The inserts were digested with NdeI and NotI-HF. The insert without a stop codon was ligated into NdeI and NotI-HF digested pET-24a(+) yielding pECTB3-MO3. The insert with a stop codon was ligated into NdeI and NotI-HF digested pET-28a(+) yielding p28CTB3-MO3.

The complete CTB2 ORF was cloned into a pCR®-Blunt vector for easier genetic manipulation. The insert was prepared by PCR using *C. nicotianae* (ATCC© 18366™) genomic DNA template with primers CTB2-5 and CTB2-3. The product was ligated into pCR®-Blunt using the Zero Blunt® PCR Cloning Kit according to the manufacturer's instructions, yielding pCR-CTB2.

Two CTB2 expression constructs were prepared. The first had a *C*-terminal 6 × His tag (pECTB2) and the second had an *N*-terminal 6 × His tag (p28CTB2). The CTB2 ORF contains two exons that were synthetically spliced using overlap extension PCR. Overlap extension PCR DNA fragments were prepared by PCR from pCR-CTB2 template. The exon 1 fragment was prepared with primers CTB2-NdeI-5 and CTB2-ex1-3. Two exon 2 fragments were prepared with primers CTB2-ex2-5 and CTB2-NotI-3 or CTB2-Stop-3, for fragments without and with a stop codon, respectively. The resulting PCR products were spliced together using overlap extension PCR with outside primers CTB2-NdeI-5 and CTB2-NotI-3 or CTB2-Stop-3 for inserts without and with a stop codon, respectively. The inserts were digested with NdeI and NotI-HF. The insert without a stop codon was ligated in NdeI and NotI-HF digested pET-24a(+) yielding pECTB2. The insert with a stop codon was ligated in NdeI and NotI-HF digested pET-28a(+) yielding p28CTB2.

Protein Expression and Purification. Proteins were prepared by heterologous expression in *E. coli* BL21(DE3) and purified by nickel-affinity purification as delineated below. Cultures used for the expression of protein constructs containing the CTB3 flavin-dependent monooxygenase domain were supplemented with 1 μM riboflavin added immediately prior to inoculation. Eluted protein fractions were dialyzed at 4 °C overnight with three buffer exchanges into 100 mM potassium phosphate pH 7.0, 10% glycerol. Purified protein concentrations were determined by the Bradford assay using bovine serum albumin (New England Biolabs) as a standard.[8] Excess protein was flash frozen in liquid nitrogen and stored at −80 °C until further use.

All proteins were expressed in *E. coli* BL21(DE3). Most protein expression constructs in this study were prepared with a T7 promoter and the lac operon for IPTG inducible expression. Overnight starter cultures were inoculated from −80 °C 20% glycerol cell stocks into LB broth supplemented with 50 μg/mL kanamycin and incubated at 37 °C with shaking at 250 rpm. Starter cultures were used to inoculate LB broth supplemented with 50 μg/mL kanamycin in baffled flasks by a 1:100 dilution. Cultures were incubated at 37 °C with shaking at 250 rpm until $OD_{600\ nm}$ 0.7 was achieved (roughly 2.5 h). The cultures were immediately submerged in ice water for 30 min. Protein expression was induced with the addition of 1 mM IPTG from a 1 M stock. Induction was carried out at 19 °C with shaking at 250 rpm for 16 h. Cells were harvested by centrifugation at 4 100 × *g* at 4 °C for 20 min. Cell pellets were flash frozen in liquid nitrogen and stored at −80 °C until further use.

In the main, proteins in these studies were purified from frozen cell pellets and harbored 6 × His tags for nickel affinity purification. Cell pellets were suspended in lysis buffer to a final concentration of 0.25 g/mL at 4 °C. Lysis buffer was composed of 50 mM potassium phosphate

pH 8.0, 300 mM NaCl, 10 mM imidazole, and 10% glycerol. The cell suspension was lysed by sonication (50% duty cycle, 10 s pulses over 3.33 min) on ice. The lysate was immediately separated by centrifugation at 25 000 × *g* at 4 °C for 20 min. High density nickel agarose beads were added to the decanted lysate and allowed to incubate at 4 °C with gentle mixing for 1 h. The nickel beads were packed into a column under gravity and washed three times with 10 column volumes of lysis buffer and two times with 10 column volumes of wash buffer at 4 °C. Wash buffer was composed of 50 mM potassium phosphate pH 8.0, 200 mM NaCl, 50 mM imidazole, and 10% glycerol. Recombinant proteins were eluted in 4 column volumes of elution buffer at 4 °C. Elution buffer was composed of 50 mM potassium phosphate pH 8.0, 300 mM NaCl, 250 mM imidazole, and 10% glycerol.

Touchdown Polymerase Chain Reaction for DNA Amplification. Standard PCR reactions were conducted using the touchdown PCR protocol.[9] Touchdown PCR reactions were prepared as 50 μL solutions of 1 × HF Buffer, 3% dimethyl sulfoxide (DMSO), 0.2 mM each dNTP, 0.2 μM each primer, 30 ng DNA template, and 1 U Phusion polymerase (New England Biolabs, Ipswich, MA). The template DNA contained the target sequence for amplification. The primers flanked the target template sequence. Touchdown PCR reactions were cycled as follows: 98 °C for 30 s; followed by 10 cycles of 98 °C for 10 s, 68 °C decreasing by 1 °C each cycle for 30 s, and 72 °C for 15 s/kbp target sequence; followed by twenty cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 15 s/kbp target sequence; followed by 72 °C for 300 s, then held at 4 °C until further use. Touchdown PCR products were run on a 1.1% agarose gel in their totality.[10] Correctly sized products were purified using a GeneJET gel extraction kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Purified products were stored at −20 °C until further use.

Overlap Extension PCR and Polymerase Chain Assembly. Overlap extension PCR was used to synthetically splice individual exons during expression construct assembly. Overlap extension PCR reactions were prepared as 50 μL solutions of 1 × HF Buffer, 3% dimethyl sulfoxide (DMSO), 0.2 mM each dNTP, 0.2 μM each outside primer, 30 ng each internal DNA fragment, and 1 U Phusion polymerase (New England Biolabs, Ipswich, MA). The internal DNA fragments contained a 20–30 bp overlap sequence at the desired splicing site. The outside primers flanked the desired total DNA target for amplification. Overlap extension PCR reactions were cycled as follows: 98 °C for 30 s; followed by 30 cycles of 98 °C for 10 s, 58 °C for 30 s, and 72 °C for 15 s/kbp target sequence; followed by 72 °C for 300 s, then held at 4 °C until further use. Overlap extension PCR products were run on a 1.1% agarose gel.[10] Correctly sized products were purified using a GeneJET gel extraction kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Purified products were stored at –20 °C until further use. Polymerase chain assembly (PCA) was used to generate entirely synthetic exons (synthons) to be used in overlap extension PCR. PCA was conducted in the same manner as overlap extension PCR with 40 bp oligonucleotides containing nesting 20 bp overlaps and no outside primers.

Assay for Flavin Identity and Content in CTB3-MO. The CTB3-MO monodomain was assayed for flavin identity and content by cofactor release through heat denaturation.[11] A 10

μM solution of CTB3-MO was incubated in a boiling water bath for 10 min. The heat-denatured sample was cooled on ice, centrifuged (14 500 × *g* at 4 °C for 10 min), and the supernatant was retained (containing free cofactor). The supernatant was also analyzed by HPLC on an Agilent 1200 fitted with a Prodigy 5u ODS3 column (4.6 × 250 mm, 5 μm, Phenomenex) using a linear gradient of 85–25% solvent A over 20 min at a flow rate of 1.0 mL/min. Solvent A was 5 mM ammonium acetate pH 6.5. Solvent B was methanol. The elution profile of released cofactor was compared to that of an authentic standard of FAD.

Corrected CTB3 and CTB2 Sequences. Both sequences for CTB3 and CTB2 were in disagreement with the previously published sequences. We report here the sequences we observe from PCR amplified genomic DNA. We assume our sequences are correct. Five corrections were made in the CTB3 nucleotide sequence: A292G, C2038A, G2075A, G2550A, and G2679A. These changes resulted in three mutations in the CTB3 protein sequence: D635N, G793D, G836E. Large sections of the CTB2 nucleotide sequence were corrected due to three separate single base insertions. The new nucleotide sequence led to a corrected CTB2 protein sequence with a new numbering scheme. The corrected CTB2 sequence showed improved alignment with other *O*-methyltransferases as determined by BLAST.

The edited CTB3 nucleotide sequence is shown below—differences in nucleotide sequence are in bold face with mutations resulting in changes in the protein sequence underlined:

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ATGATGCAGTTCCAACGCGATCTTGAGGCGTCCTTGGAGGCCGTATCGGCCAACGCCCAGGAGC
TGCTCAAATCTCTCAAGAGTCGCAAGGATGTTCAAGACCTCAACGCGTCGTTGCCGAAGGATCC
TTTAGACAACTGCGATGCTCAAACTCAAGCCGCTCGTGCGCAGCTGGCAGAGGCAGCGACAAGA
ATCTTGCAGTTGTCGATCCGACCTCAAGAGTATCTGGAGCATCTACAAAACGGCTATCAACATT
TAACCTGTTTTCGCTGGCTGGTGGAACTCAACATATTGGACCACCTTCCACATAGCGGAACGAT
CAGCTACACAGATCTTGCGAGAAAAGCCAGCGTGCCGCCTATGCAATTGAGAAGCATCTGTCGC
ATGGCCATATGCAATGGATTCCTGGAAGAGCCCGAGGCCAACCAAGTCCGCCACAGTCGCATTT
CCGCCTTGTTCGCTCGCGATGAAAGCTATTTAGGTTGGGCTAGATGGATGGTCAACTACTCTGT
GCCAGCTGCATACAAGCTTAGCGACGCCACGCGATCGTGGGGCGAGACTGTCGCCAAAGATCAG
ACCGCGTTCAATCTGGGAATGGATGTGAAAGTCCCATTCTTTGACCATCTCCGCCAGACGCCCG
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CAATGAAGGACGCCTTTGCAGCTTATATGCGTAATGTGACTTCGAACGCAACTTGGGGCCTCCA
GCACGCAGTCACCGGCTTCGACTGGGCTTCCCTTCCGCGGGGCGCAAAAGTCGTGGATGTCGGT
GGCTCTCTTGGGCATGGTAGCATTGCCATTGCCAAGGAGCACACTCACCTTACCTTCGTCATTC
AAGATCTGCCAGAGACGGTCGCTGGTGCCAGGAAAGAAATGGCCCAAAATGACAAGATTGAAGC
TTCTGTTAAATCTCGCATCACCTTTCAGGAACATGACTTCTTTGGTCCTCAAACAGTGAAGGAT
GCCGATGTTTACTTTCTTCGCATGATCTGTCACGACTGGCCCGACAACGAAGCCAAGGTCATCC
TCTCTCAGATTCGCGCTGCACTGAAACCTGGGGCGCAAATAGTCATCATGGACACCATTCTTCC
CCAGCCCGGCACAATTAGCGTTTTGCAAGAGCAACAACTACGCATTCGGGATCTAACAATGATG
GAAGTCTTCAATGCCAAGGAGCGTGAATTGGAGGACTGGAGCTCATTGATGCAATCTGCCGGTC
TCGAGATTTCTCGCGTGAACCAGCCGCTCAACAGTGTGATGGGTCTGCTCACAGTCCGCTCAGC
CGGCCAAACTGCCCTCTCCGGAACGAATACACTGACGCCAGAGTTGGTGGCGGCAGTCTCCGCA
AGCACTGGCTCTGCTGATTCGAGGCCAGTCCTCATCGCAGGCGCGGGTATTGCTGGGCTCTGCC
TTGCACAGGCTTTGAAGAAGGCCGGCATTGACTTTCGCGTCTTCGAAAGGGACTTCCATGTCGA
TGCTCGGCCACAAGGATACCGACTCAAATTCGAAGCAGACGCCGCACAGTCTCTCAAGAACATC
CTGCCTGACGATGTTTATGAGGCTTTCGAACTGTCAAATGCCGTCACCGCCGTAGGCGAGACGG
ACTTCAATCCCTTCAATGGTAACATAATCCACAGCCGCACTGGTGGCGGCCTGTCTGGCAAGAA
GGGACTGTATGCGACATTCACTGTTGACCGCAAAGCATTCAGAACTCAGCTCATGACTGGCATT
GAGGACAAGATTTCGTTCGGGAAGGAAATCGCGTACTACAAGACCGATGACGCTACATCTACGG
TCAACGCAGAATTCAAGGACGGCACTCACGTCACCGGAAGTTTCCTGGCCGGCACTGATGGCTT
ACACTCTGTAGTTCGCAAGACATGTGTACCAAACCATCGTATCGTG**A**ATACTGGTGCTGCCTGC
ATCTACGGCAAGACTGTAATGACACCGGAATTCCTCGCGCGATTCCCCGAGAAAGGCTTGAGAT
TCATGACAGTGGTCAGCGACATCGCACCTATGCTACAGTCTTGTCTCATCGGCGACAGCCCAGT
CACCTTACTACTGGAGCCTATCCGATTCAGCGAAGCCTCGCGTGCCCGCTATCCAGAACTGCCT
CCAGACTACGTCTACTGGGCCCTCATCGGACCCAAGGAACGTTTCGGATCGCAAGAGGTGACTT
CCATGAAGAACTTCGTCTCACTGGACCAAGCGGCAGAACAGGCTGCCAAGCTCAGTCTCGCAGT
CACCGAGGAATGGCATCCGAGCCTTCGCGCGCTGTTTGAGCTTCAAGACACGAAGCAAGCATCG
CTCATTCGCGTTGCATCCACAATTCCCGATATCCCATCTTGGGAGTCCCACTCCAATGTTACCG
TTCTTGGCG**A**TAGCATTCATCCGATGAGCCCTTGTGGTGGAGTCGGAGCGAACACCGCAATAGT
CGATGCCGACGCCTTGGCTAAAGTGCTCGTTGAGCATGGTACGAAGCCACCGGTGAACGCAATC
GCCGAGTTCG**A**GGCCGCGATGAGAACGAGAGCGAAGAGGAACATTTGGAGGAGTGAGGTTGGCA
GCAAGAGGATGTTTGGACAGAAGAATCTGGTGGATTGTTCAGAGTTTGTGTTTTGA

The edited CTB2 nucleotide sequence is shown below—differences in nucleotide sequence are in bold face with mutations resulting in changes in the protein sequence underlined and insertions italicized:

ATGGTTAAACGAATCGAAGCGGACAATCTCTTTGAGCTCACGGCAGAGCTGGTCTCAGCCTCCT
CAAAGCTTCACAAATTTCTAGACCAGAAGAACCTTCCGCAGCCATCGTTTGATGCTCCAGCTCC
ATCGGTTGCTCTCAACTCAGCCAACAAGCCGTACTATGATGCGAGAAGCGCGATTGTAGAGGCT
GCTGAGCAACTCATTCGCCTGGTCCGTGGACCTCGCGACACACTCCTCGCTCTGTCTTTCGAGC
ACTGCGCTACGGCGTCGATGCAGGTCGTCTTCAAATACAAGTTTGCGAATCACATCCCGCTACA
TGGCTCGACAACTTATAGCAAGATTGCCGAAGCAGTCGGAGATGGTGTGACAACAGCTCTCGTT
GAGCGCACGATACAACATTGCGCTTCCTTTGGCCTATTCGAGACGATCCCTGGCG***G***CTATGTTA
CTC***A***CAATGCTACCTCGTC***A***CTACTGGTCACCGATCCAGATCTCGAAGCCTGGATGTATCTCTC
GGCGGTGATAGCCTACCCAGCTGGCGCAGCTATCCCTAAGGCTGTAGAGCAGTATGGCGTTTCC

CATGAAGCTGACGAATCAGGGTACGGTGCCAGCATAGGAAGAAAGATTGCACAATTCCAGCGAT
TCCGTGAGCCCGATGGGAAGAAGGACCACGAGATGTTCGCACGAGCCATGCGTGGTATCGCGGC
TGGTGGTGCGTATGACTTCCGCCATGCAGTCGATGGCGGATACCCTTGGCACCTCCTCGCAGAG
GGCGCAGGTCACCTGGTCGTGGATGTTGGTGGAGGTCCCGGCCACGTCGCCATGGCACTCGCGG
AAAAGTACCCAAGCTTGCGTTTCCAAGTCCAAGATCTGCCCGAGACCGTCCAAGTGGGAGCGAA
GAATTGCCCTGAGCACTTGAAGTCCCGCGTGTCATTCCAGAGCCACGATTTCTTCACCTCGCAA
CCTGCTCATGAAGTGCAAGACGGCGAAGGAATCGTCTACTTTGCTCGATTCATCTTGCACGACT
GGAGTGACAAGTATGCCACCAAGATCGTGCAGCAACTTGCAACTGGCTTGAGGCCACAAGATCG
CATCATTTTGAACGAAGTGGTCGTTCCCGAAGCCGGACAAGTTGGCAGAGAGACCGAACGAAGA
ATGCACGATCGTGATCTGCTGATGTTGATGAACCTCAACGGCCGTGAGCGGACACAGAGTGCAT
TCGAGGCGATCTTCGCTTCAGTGACTCCCAAGCTGCGGCTGCAGAGGGTCATTCACCCAGAGCA
AGGCGAATTGTCGCTTATCGAGGTGACTCTTGATGGCGTTGAGCTTCCTGCCCAGGCTAATGGC
GTCAACGGCCATGCGAATGGCACCAATGGTGTCAATGGTCA**G**TAA

The edited CTB3 protein sequence is shown below—mutations are in boldface and underlined:

MMQFQRDLEASLEAVSANAQELLKSLKSRKDVQDLNASLPKDPLDNCDAQTQAARAQLAEAATR
ILQLSIRPQEYLEHLQNGYQHLTCFRWLVELNILDHLPHSGTISYTDLARKASVPPMQLRSICR
MAICNGFLEEPEANQVRHSRISALFARDESYLGWARWMVNYSVPAAYKLSDATRSWGETVAKDQ
TAFNLGMDVKVPFFDHLRQTPAMKDAFAAYMRNVTSNATWGLQHAVTGFDWASLPRGAKVVDVG
GSLGHGSIAIAKEHTHLTFVIQDLPETVAGARKEMAQNDKIEASVKSRITFQEHDFFGPQTVKD
ADVYFLRMICHDWPDNEAKVILSQIRAALKPGAQIVIMDTILPQPGTISVLQEQQLRIRDLTMM
EVFNAKERELEDWSSLMQSAGLEISRVNQPLNSVMGLLTVRSAGQTALSGTNTLTPELVAAVSA
STGSADSRPVLIAGAGIAGLCLAQALKKAGIDFRVFERDFHVDARPQGYRLKFEADAAQSLKNI
LPDDVYEAFELSNAVTAVGETDFNPFNGNIIHSRTGGGLSGKKGLYATFTVDRKAFRTQLMTGI
EDKISFGKEIAYYKTDDATSTVNAEFKDGTHVTGSFLAGTDGLHSVVRKTCVPNHRIV**N**TGAAC
IYGKTVMTPEFLARFPEKGLRFMTVVSDIAPMLQSCLIGDSPVTLLLEPIRFSEASRARYPELP
PDYVYWALIGPKERFGSQEVTSMKNFVSLDQAAEQAAKLSLAVTEEWHPSLRALFELQDTKQAS
LIRVASTIPDIPSWESHSNVTVLG**D**SIHPMSPCGGVGANTAIVDADALAKVLVEHGTKPPVNAI
AEF**E**AAMRTRAKRNIWRSEVGSKRMFGQKNLVDCSEFVF

The edited CTB2 protein sequence is shown below—mutations are in boldface and underlined:

MVKRIEADNLFELTAELVSASSKLHKFLDQKNLPQPSFDAPAPSVALNSANKPYYDARSAIVEA
AEQLIRLVRGPRDTLLALSFEHCATASMQVVFKYKFANHIPLHGSTTYSKIAEAVGDGVTTALV
ERTIQHCASFGLFETIPG**GYVTHNATSS**LLVTDPDLEAWMYLSAVIAYPAGAAIPKAVEQYGVS
HEADESGYGASIGRKIAQFQRFREPDGKKDHEMFARAMRGIAAGGAYDFRHAVDGGYPWHLLAE
GAGHLVVDVGGGPGHVAMALAEKYPSLRFQVQDLPETVQVGAKNCPEHLKSRVSFQSHDFFTSQ
PAHEVQDGEGIVYFARFILHDWSDKYATKIVQQLATGLRPQDRIILNEVVVPEAGQVGRETERR
MHDRDLLMLMNLNGRERTQSAFEAIFASVTPKLRLQRVIHPEQGELSLIEVTLDGVELPAQANG
VNGHANGTNGVNG**Q**

***In Vitro* Reactions of CTB3.** *Nor*-toralactone and toralactone purified from the ΔCTB3c mutant strain were used as substrates for *in vitro* reactions of CTB3. Stock solutions of the substrates (2 mM) were prepared in 90% dimethyl sulfoxide (DMSO) [*aq*.] and stored at −80 °C until use. NADH stock solutions (50 mM) were prepared immediately before use in 100 mM potassium phosphate pH 7.0, 10% glycerol. Dithiothreitol (DTT) and *S*-adenosyl-L-methionine (SAM) stock solutions (100 mM and 50 mM, respectively) were prepared in 100 mM potassium phosphate pH 7.0, 10% glycerol and stored at −20 °C until use. Reactions were carried out in potassium phosphate pH 7.0, 10% glycerol. Reaction mixtures were prepared by diluting CTB3 (intact, CTB3-MT, CTB3-MO, or CTB2, 1.0 μM final concentration) in buffer followed by the addition of cosubstrates (NADH or SAM, as needed; 1 mM final concentration), followed by the addition of DTT, as needed (1 mM final concentration). Reactions were initiated with the addition of naphthopyrone substrate (0.1 mM final concentration, 4.5% DMSO final concentration) and immediately mixed by gentle agitation. Reactions were allowed to proceed at room temperature for 1 h, and then were quenched with the addition of 5 *N* HCl (5 μL to 250 μL reaction). The reaction mixtures were filtered through 0.2 μm PTFE filters and analyzed by HPLC. HPLC was conducted on an Agilent 1200 system fitted with a diode array detector (DAD) and a Kinetex XB-C18 column (4.6 × 75 mm, 2.6 μm). The gradient method was as follows: 5–95% acetonitrile + 0.1% formic acid, over 10.8 min, at a flow rate of 1.25 mL/min.

Chemical Characterization of Cercosporin Pathway Products. ^{1}H NMR data were collected on a 400 MHz Bruker Avance spectrometer (Bruker, Billerica, MA). HRMS data were collected on a Waters Acquity/Xevo-G2 UPLC-MS system (Waters, Milford, MA). MS and MS/MS spectra were collected in positive ion mode. HPLC was conducted on an Agilent 1200

system fitted with a DAD and a Kinetex XB-C18 column (4.6 × 75 mm, 2.6 μm). UV data were determined from the DAD signal at the maximum HPLC chromatograph signal. Spectra are presented in the Supporting Information (Figure S5–18).

5,12-dihydroxy-8,9-bis((*R*)-2-hydroxypropyl)-7,10-dimethoxyperyleno[1,12-*def*][1,3]dioxepine-6,11-dione (cercosporin, 1). Cercosporin was purified from wild type *C. nicotianae* (ATCC© 18366™) as a red powder. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.07 (s, 2H), 5.74 (s, 2H), 4.21 (s, 6H), 3.59 (dd, *J* = 12.8, 6.8 Hz, 2H), 3.39 (m, 2H), 2.90 (dd, *J* = 12.8, 6.0 Hz, 2H), 0.65 (d, *J* = 6.0 Hz, 6H); HRMS–ESI/ACPI-TOF (*m*/*z*): [MH^+] calcd for $C_{29}H_{27}O_{10}$, 535.1604; found, 535.1599 (100); UV (acetonitrile [*aq*.]) $\lambda_{max,}$ nm: 224, 260, 272, 324, 470, 566; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 7.22 min. These data are in agreement with literature values for cercosporin.[12]

7,9,10-trihydroxy-3-methyl-1*H*-benzo[*g*]isochromen-1-one (*nor*-toralactone, 6). *Nor*-toralactone was purified from *C. nicotianae* ΔCTB3c as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 13.61 (s, 1H), 9.50 (s, 1H), 6.92 (s, 1H), 6.61 (d, *J* = 2.4 Hz, 1H), 6.48 (d, *J* = 2.4 Hz, 1H), 6.23 (s, 1H), 2.27 (s, 3H); HRMS–ESI/ACPI-TOF (*m*/*z*): [MH^+] calcd for $C_{14}H_{11}O_5$, 259.0607; found, 259.0606 (100); UV (acetonitrile [*aq*.]) λ_{max}, nm: 268, 278, 292, 390; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 6.83 min. These data are in agreement with literature values for *nor*-toralactone.[13]

9,10-dihydroxy-7-methoxy-3-methyl-1*H*-benzo[*g*]isochromen-1-one (toralactone, 7). Toralactone was purified from *C. nicotianae* ΔCTB3c as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 13.55 (s, 1H), 9.42 (s, 1H), 6.98 (s, 1H), 6.62 (d, *J* = 2.4 Hz, 1H), 6.54 (d, *J* = 2.4 Hz, 1H), 6.24 (s, 1H), 3.90 (s, 3H), 2.27 (s, 3H); HRMS–ESI/ACPI-TOF (*m*/*z*): [MH^+] calcd for $C_{15}H_{13}O_5$, 273.0763; found, 273.0764 (100); UV (acetonitrile [*aq*.]) λ_{max}, nm: 268, 278, 292, 390;

HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 8.51 min. These data are in agreement with literature values for toralactone.[14]

7,9-dihydroxy-3-methyl-5-(λ³-oxidanylidene)-1*H*-benzo[*g*]isochromene-1,10(5*H*)-dione (8). Product **8** was purified from *C. nicotianae* ΔCTB3c as a red powder. This product was also observed as an oxidation product of *nor*-toralactone in *in vitro* CTB3 reactions. HRMS–ESI/ACPI-TOF (*m/z*): [MH^+] calcd for $C_{14}H_9O_6$, 273.0399; found, 273.0388 (100); UV (acetonitrile [*aq.*]) λ_{max}, nm: 248, 270sh, 296sh, 474; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 4.92 min.

2,5-dihydroxy-6-methoxy-7-(2-oxopropyl)naphthalene-1,4-dione (cercoquinone A, 9). Metabolite **9** was purified from *C. nicotianae* ΔCTB6 as an orange powder. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 12.76 (s, 1H), 7.49 (s, 1H), 6.26 (s, 1H), 4.05 (s, 3H), 3.80 (s, 2H), 2.27 (s, 3H); HRMS–ESI/ACPI-TOF (*m/z*): [MH^+] calcd for $C_{14}H_{13}O_6$, 277.0712; found, 277.0711 (100); HRMS/MS–ESI/ACPI-TOF (*m/z*): [MH^+ –CO] calcd for $C_{13}H_{13}O_5$, 249.0763; found, 249.0756 (15); [MH^+ –CO –H_2O] calcd for $C_{13}H_{11}O_4$, 231.0657; found, 231.0649 (17); [MH^+ –C_3H_5O] calcd for $C_{11}H_8O_5$, 220.0372; found, 220.0369 (54); [MH^+ –$C_2H_5O_2$] calcd for $C_{12}H_8O_4$, 216.0423; found, 216.0415 (30); [MH^+ –CO –H_2C=C=O] calcd for $C_{11}H_{11}O_4$, 207.0657; found, 207.0655 (100); [MH^+ –$C_3H_4O_2$] calcd for $C_{11}H_9O_4$, 205.0501; found, 205.0494 (42); [MH^+ –$C_4H_5O_2$] calcd for $C_{10}H_8O_4$, 192.0423; found, 192.0414 (57); [MH^+ –$C_4H_6O_2$] calcd for $C_{10}H_7O_4$, 191.0344; found, 191.0343 (43); [MH^+ –$C_3H_4O_3$] calcd for $C_{11}H_9O_3$, 189.0552; found, 189.0546 (63); [MH^+ –$C_4H_4O_3$] calcd for $C_{10}H_9O_3$, 177.0552; found, 177.0543 (35); [MH^+ –$C_4H_6O_3$] calcd for $C_{10}H_7O_3$, 175.0395; found, 175.0390 (79); [MH^+ –$C_4H_4O_4$] calcd for $C_{10}H_9O_2$, 161.0603; found, 161.0594 (47); [MH^+ –$C_6H_6O_3$] calcd for $C_8H_7O_3$, 151.0395; found, 151.0388 (21); UV

(acetonitrile [*aq*.]) λ_{max}, nm: 248, 300, 422; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 5.42 min.

9-hydroxy-6-methoxy-2-methyl-2,3-dihydronaphtho[2,3-*b*]furan-5,8-dione (cercoquinone B, 10). Metabolite **10** was purified from *C. nicotianae* ΔCTB5 as a red-orange powder. ^{1}H NMR (400 MHz, CD_3CN, δ): 7.55 (s, 1H), 6.19 (s, 1H), 3.97 (s, 3H), 2.78 (m, 2H), 1.16 (d, J = 6.4 Hz, 3H); HRMS–ESI/ACPI-TOF (*m/z*): [MH^+] calcd for $C_{14}H_{13}O_5$, 261.0763; found, 261.0761 (100); HRMS/MS–ESI/ACPI-TOF (*m/z*): [$MH^+ - CH_3$] calcd for $C_{13}H_{10}O_5$, 246.0528; found, 246.0529 (36); [$MH^+ - CO$] calcd for $C_{13}H_{13}O_4$, 233.0814; found, 233.0812 (37); [$MH^+ - 2CH_3$] calcd for $C_{12}H_7O_5$, 231.0293; found, 231.0295 (37); [$MH^+ - CH_3 - H_2O$] calcd for $C_{13}H_8O_4$, 228.0423; found, 228.0419 (22); [$MH^+ - C_3H_4O$] calcd for $C_{11}H_9O_4$, 205.0501; found, 205.0499 (46); [$MH^+ - C_3H_6O$] calcd for $C_{11}H_7O_4$, 203.0344; found, 203.0345 (100); [$MH^+ - CO - CH_3OH$] calcd for $C_{12}H_9O_3$, 201.0551; found, 201.0531 (26); [$MH^+ - C_2H_5O_2$] calcd for $C_{12}H_8O_3$, 200.0473; found, 200.0474 (68); [$MH^+ - C_3H_5O_3$] calcd for $C_{11}H_8O_2$, 172.0534; found, 172.0523 (25); UV (acetonitrile [*aq*.]) λ_{max}, nm: 250, 302, 426; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 5.07 min.

3,5-dihydroxy-7-methoxy-2-(2-oxopropyl)naphthalene-1,4-dione (cercoquinone D, 11). 1,4-Naphthoquinone **11** was analyzed from *in vitro* reactions of CTB3 against *nor*-toralactone or toralactone as a pale yellow residue. Limited yields excluded ^{1}H NMR analysis of this product. HRMS–ESI/ACPI-TOF (*m/z*): [MH^+] calcd for $C_{14}H_{13}O_6$, 277.0712; found, 277.0717 (100); HRMS/MS–ESI/ACPI-TOF (*m/z*): [$MH^+ - H_2C{=}C{=}O$] calcd $C_{12}H_{11}O_5$, 235.0607; found, 235.0615 (100); [$MH^+ - CO - H_2O$] calcd $C_{13}H_{11}O_4$, 231.0657; found, 231.0656 (24); [$MH^+ - H_2C{=}C{=}O - H_2O$] calcd $C_{12}H_9O_4$, 217.0501; found, 217.0502 (65); [$MH^+ - H_2C{=}C{=}O - H_2O - CO$] calcd $C_{11}H_9O_3$, 189.0551; found, 189.0558 (51); [$MH^+ - H_2C{=}C{=}O - H_2O$

–2CO] calcd $C_{10}H_9O_2$, 161.0602; found, 161.0611 (42); [MH^+ –CO –$C_5H_6O_2$] calcd $C_8H_7O_3$, 151.0395; found, 151.0401 (46); UV (acetonitrile [*aq*.]) λ_{max}, nm: 260, 320sh, 380sh; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 4.65 min.

8-hydroxy-6-methoxy-3-(2-oxopropyl)naphthalene-1,2-dione (cercoquinone C, 12). 1,2-Naphthoquinone **12** was analyzed from reactions of CTB3 under non-reductive conditions. Limited yields excluded ^{1}H NMR analysis of this product. HRMS–ESI/ACPI-TOF (*m/z*): [MH^+] calcd for $C_{14}H_{13}O_5$, 261.0763; found, 261.0767 (100); [MNa^+] calcd for $C_{14}H_{12}O_5Na$, 283.0582; found, 283.0582 (76); HRMS/MS–ESI/ACPI-TOF (*m/z*): [MH^+ –CO –H_2O] calcd $C_{13}H_{11}O_3$, 215.0708; found, 215.0709 (100); [MH^+ –H_2C=C=O –CO] calcd $C_{11}H_{11}O_3$, 191.0708; found, 191.0708 (91); [MH^+ –2CO –H_2O] calcd $C_{12}H_{11}O_2$, 187.0759; found, 187.0757 (92); [MH^+ –2CO –H_2O –CH_3] calcd $C_{11}H_8O_2$, 172.0524; found, 172.0522 (56); [MH^+ –H_2C=C=O –2CO] calcd $C_{10}H_{11}O_2$, 163.0759; found, 163.0758 (31); [MH^+ –CO –C_5H_6O] calcd $C_8H_7O_3$, 151.0395; found, 151.0399 (28); [MH^+ –H_2C=C=O –2CO –H_2O] calcd $C_{10}H_9O$, 144.0653; found, 144.0576 (77); UV (acetonitrile [*aq*.]) λ_{max}, nm: 216, 286, 306sh, 386, 504; HPLC (Kinetex XB-C18, 1.25 mL/min, 5–95% acetonitrile + 0.1% formic acid, 10.8 min) t_R = 6.39 min.

Table S1. Primers used to clone new constructs in this study. Underlined sequences align to adjacent exons. Bold sequences are introduced restriction enzyme cut sites. Italicized sequences are introduced stop codons.

Primer	Sequence
CTB3-5	ATGATGCAGTTCCAACGCGATCTTGAGGCG
CTB3-3	TCAAAACACAAACTCTGAACAATCCACCAG
CTB3-AseI-5	GCCG**ATTAAT**ATGATGCAGTTCCAACGCGAT
CTB3-NotI-3	ATTA**GCGGCCGC**AAACACAAACTCTGAACAATC
CTB3-Stop-3	ATTA**GCGGCCGC***TCA*AAACACAAACTCTGAACA
CTB3-ex1-3	ATGTTGATAGCCGTTTTGTAGATGCTCCAG
CTB3-ex2-5	CAAAACGGCTATCAACATTTAACCTGTTTT
CTB3-ex2-3	GTAGTTGACCATCCATCTAGCCCAACCTAA
CTB3-ex3-5	AGATGGATGGTCAACTACTCTGTGCCAGCT
CTB3-MT3-3	ATTA**GCGGCCGC**GGAGAGGGCAGTTTGGCCGGCTGA
CTB3-MT3-Stop-3	ATTA**GCGGCCGC***TTA*GGAGAGGGCAGTTTGGCCGGCTGA
CTB3-MO1-5	ATTA**CATATG**GATTCGAGGCCAGTCCTCATCGCA
CTB2-5	ATGGTTAAACGAATCGAAGCGGACAATCTC
CTB2-3	TTAATGACCATTGACACCATTGGTGCCATT
CTB2-NdeI-5	ATTA**CATATG**GTTAAACGAATCGAAGCG
CTB2-NotI-3	ATTA**GCGGCCGC**ATGACCATTGACACCATTGGT
CTB2-Stop-3	ATTA**GCGGCCGC***TTA*ATGACCATTGACACCATT
CTB2-ex1-3	GAAGACGACCTGCATCGACGCCGTAGCGCA
CTB2-ex2-5	TCGATGCAGGTCGTCTTCAAATACAAGTTT

Table S2. Details of expression constructs used in this study.

Protein	Encodes	Plasmid	Vector	Tag	MW (kDa)
CTB3	M1–F871[a]	p28CTB3-3	pET-28a(+)	N-His	98.2
CTB3-MT	M1–S433	p28CTB3-MT3-3	pET-28a(+)	N-His	50.7
CTB3-MO	D454–F871[a]	pECTB3-MO1-3	pET-24a(+)	C-His	47.0
CTB2	M1–Q462[b]	pECTB2-1	pET-24a(+)	C-His	51.8

[a] New CTB3 protein sequence as described above.
[b] New CTB2 protein sequence as described above.

RESULTS

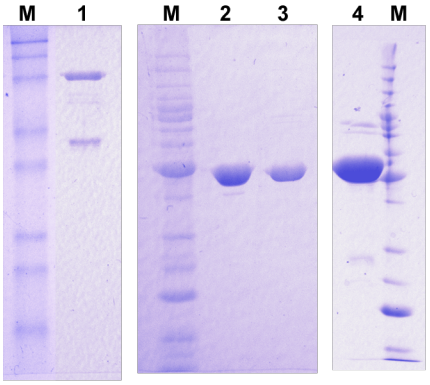


Figure S1. 12% SDS-PAGE analysis of proteins used in this study. Lane M: Ladder; 1: CTB3; 2: CTB3-MT; 3: CTB3-MO; 4: CTB2.

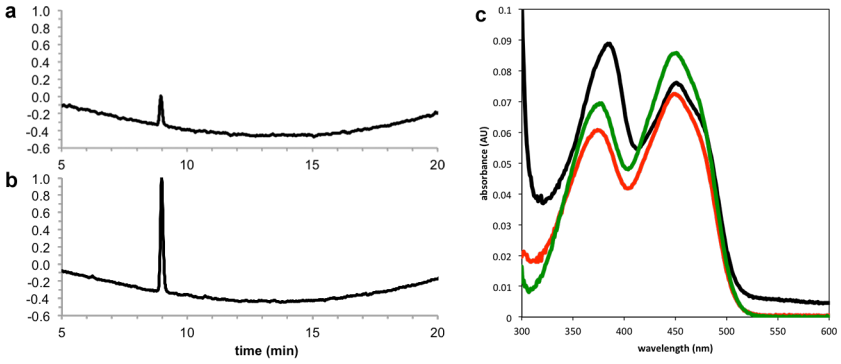


Figure S2. Analysis of FAD content of CTB3-MO. (a) HPLC analysis of FAD released from heat denatured CTB3-MO. (b) HPLC analysis of FAD standard. (c) UV-vis spectra of CTB3-MO (black), FAD released from heat denatured CTB3-MO (red), and free FAD (green).

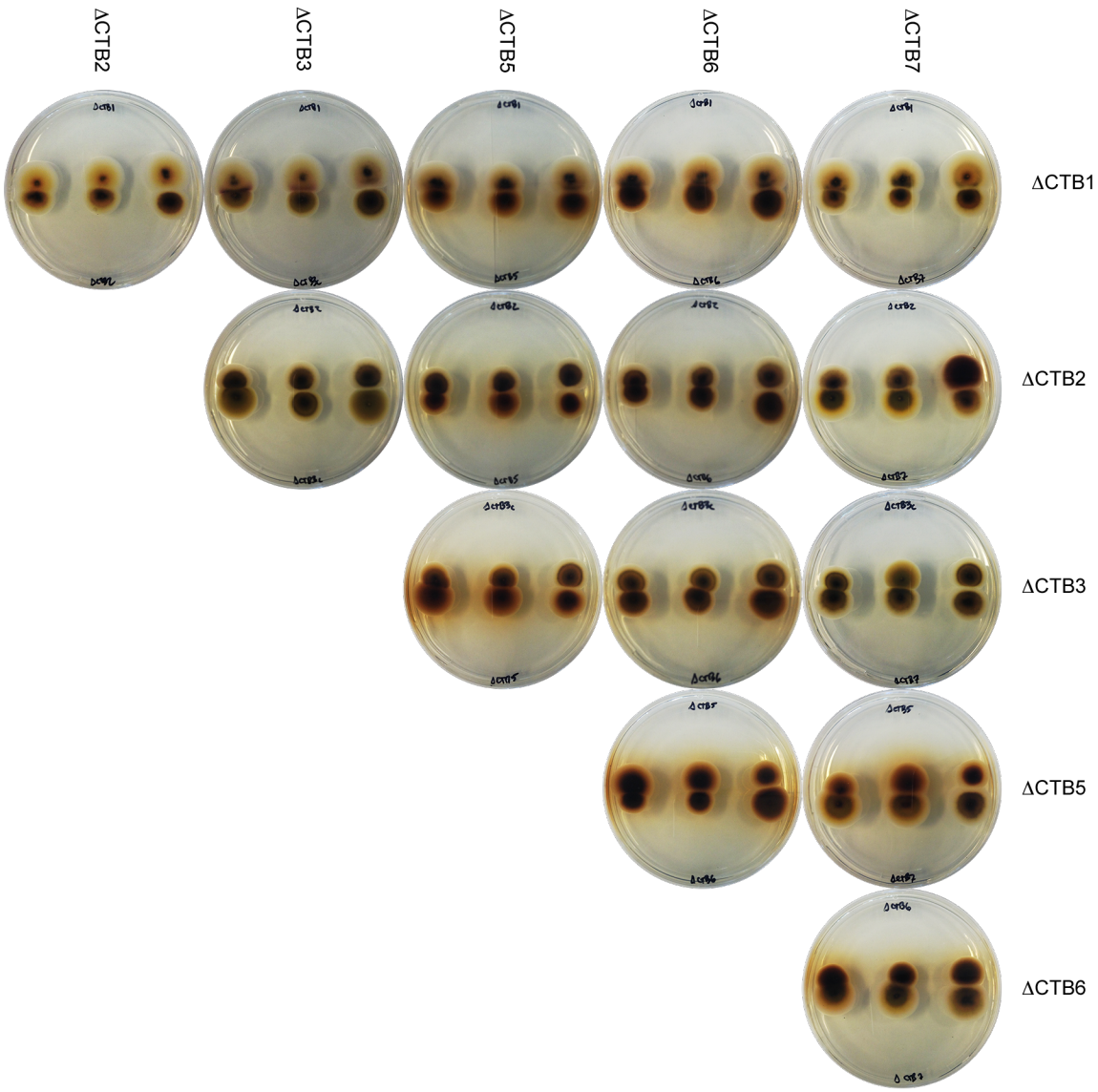


Figure S3. Pairwise complementation assay of *C. nicotianae* cercosporin biosynthetic gene knockout strains. Labels for each row indicate the identity of the top colony on each plate. Labels for each column indicate the identity of the bottom colony on each plate. Cercosporin complementation is indicated by the presence of a red pigment at the colony–colony interface. Colonies are spaced 0.5, 0.7, and 1.0 cm apart, from left to right.

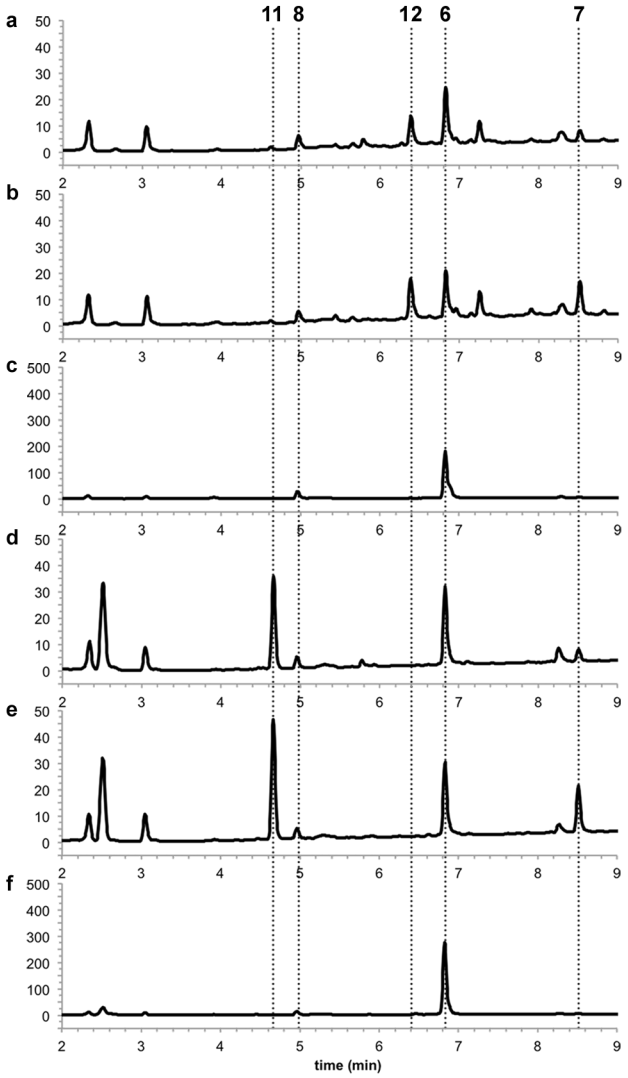


Figure S4. Product profiles of *in vitro* reactions of CTB3 and CTB2. The 280 nm HPLC chromatograms of the following reactions are displayed: (a) CTB3-MT, CTB3-MO, CTB2 with *nor*-toralactone, (b) CTB3-MT, CTB3-MO with *nor*-toralactone, (c) CTB2 with *nor*-toralactone, (d) CTB3-MT, CTB3-MO, CTB2 with *nor*-toralactone under reductive conditions, (e) CTB3-MT, CTB3-MO with *nor*-toralactone under reductive conditions, (f) CTB2 with *nor*-toralactone under reductive conditions.

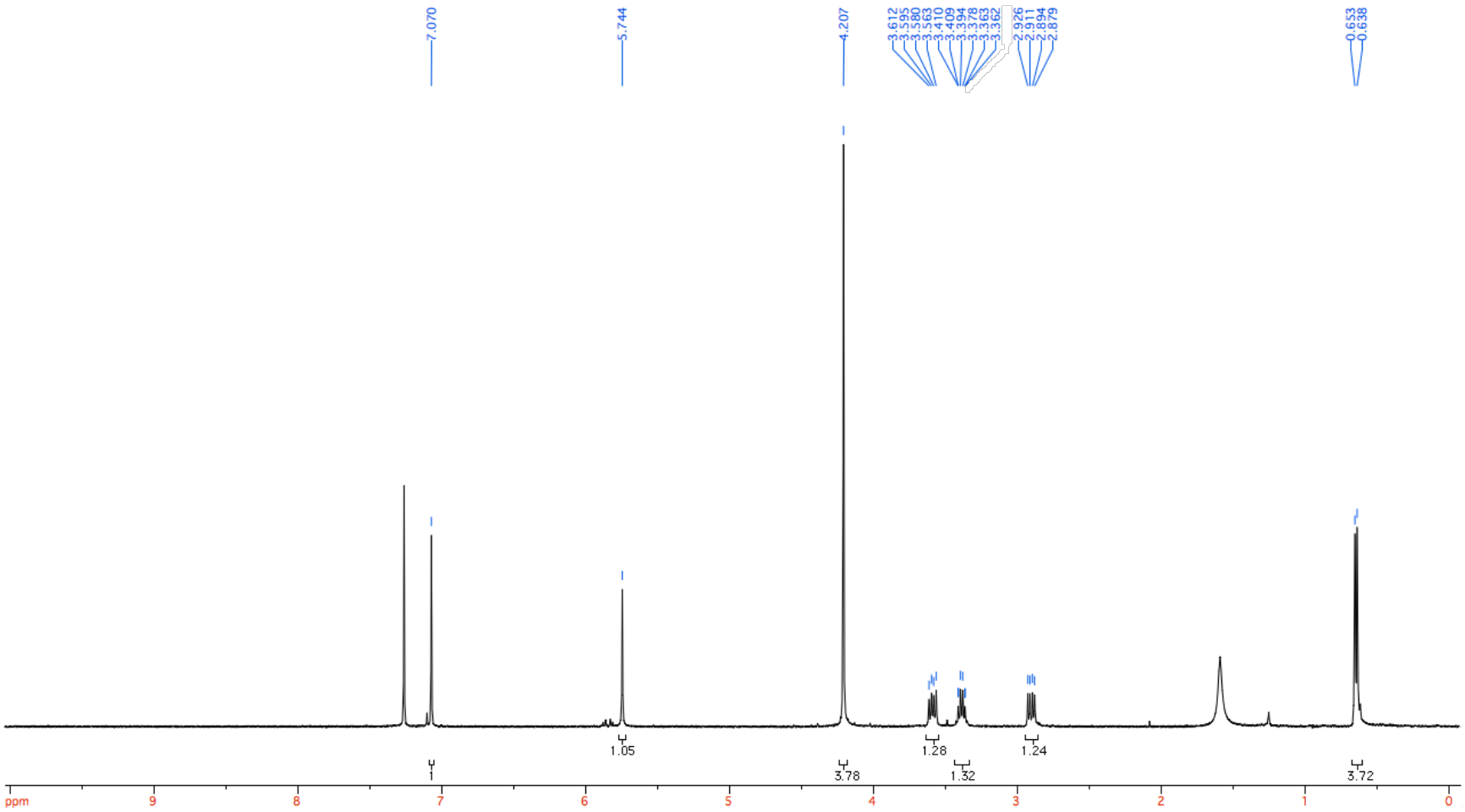


Figure S5. ^{1}H NMR (400 MHz, $CDCl_3$) of cercosporin (**1**) purified from *C. nicotianae* (ATCC© 18366™).

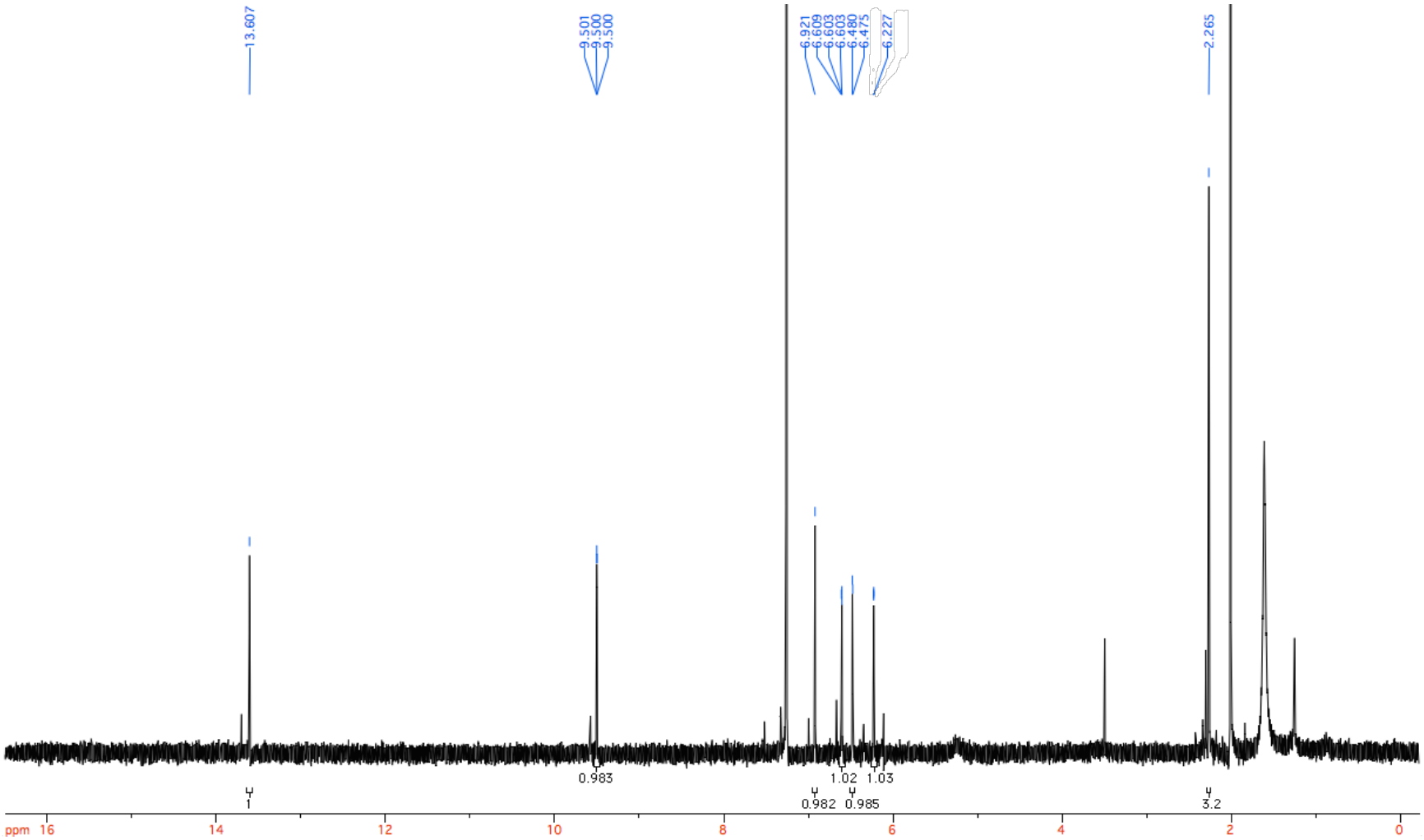


Figure S6. ^{1}H NMR (400 MHz, $CDCl_3$) of *nor*-toralactone (**6**) purified from *C. nicotianae* ΔCTB3c.

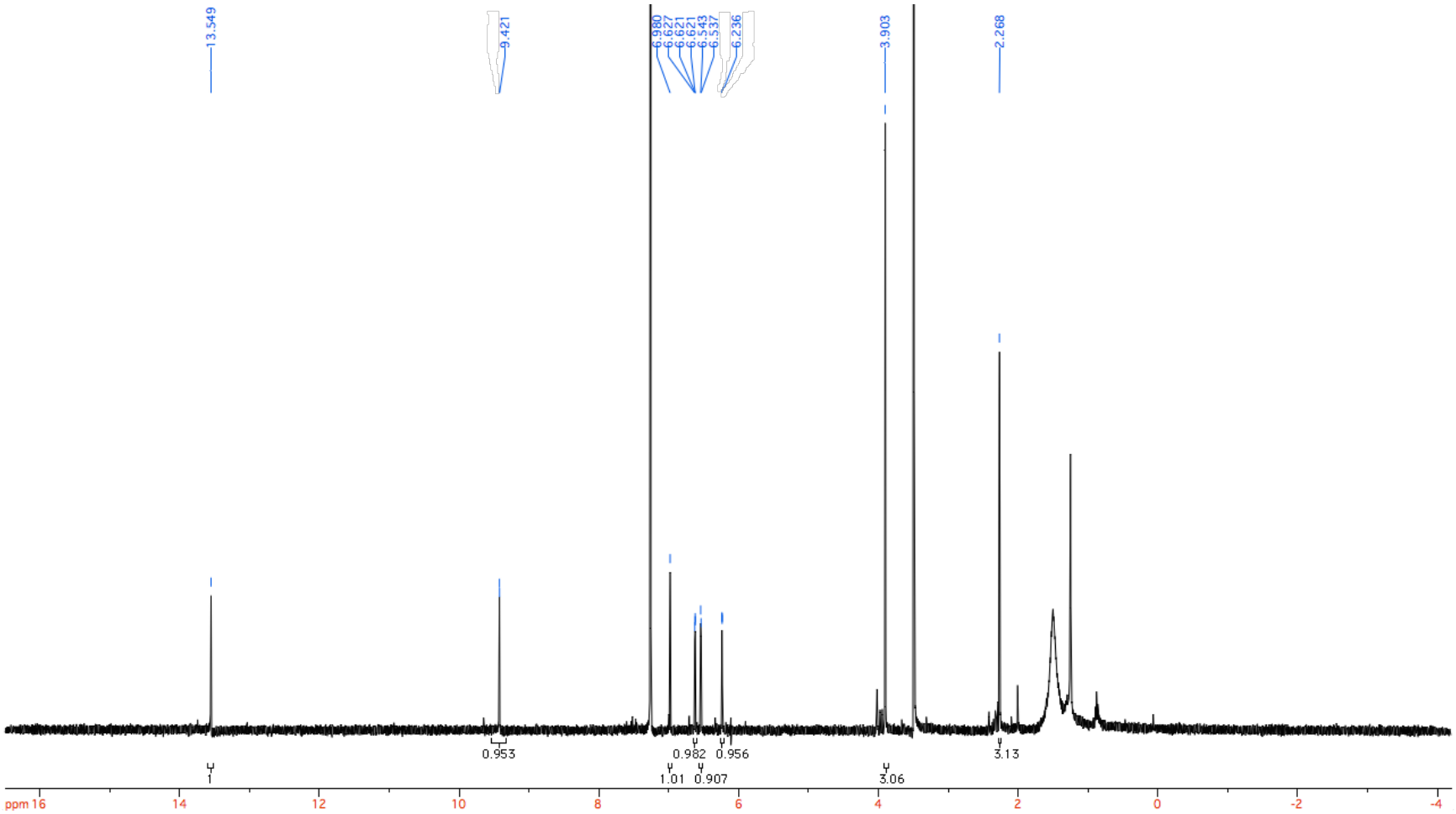


Figure S7. ^{1}H NMR (400 MHz, $CDCl_3$) of toralactone (**7**) purified from *C. nicotianae* ΔCTB3c.

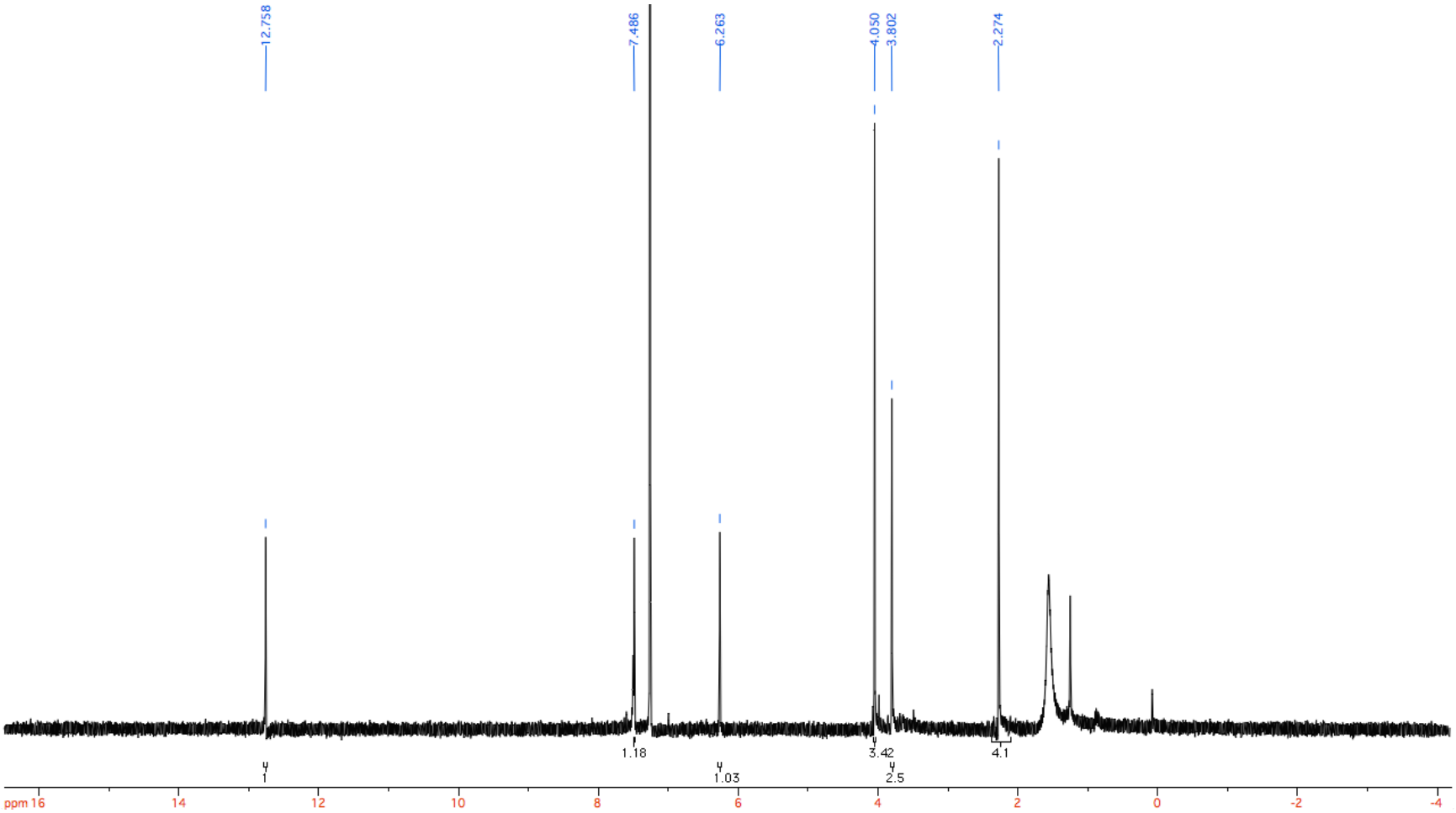


Figure S8. ^{1}H NMR (400 MHz, $CDCl_3$) of cercoquinone A (**9**) purified from *C. nicotianae* ΔCTB6.

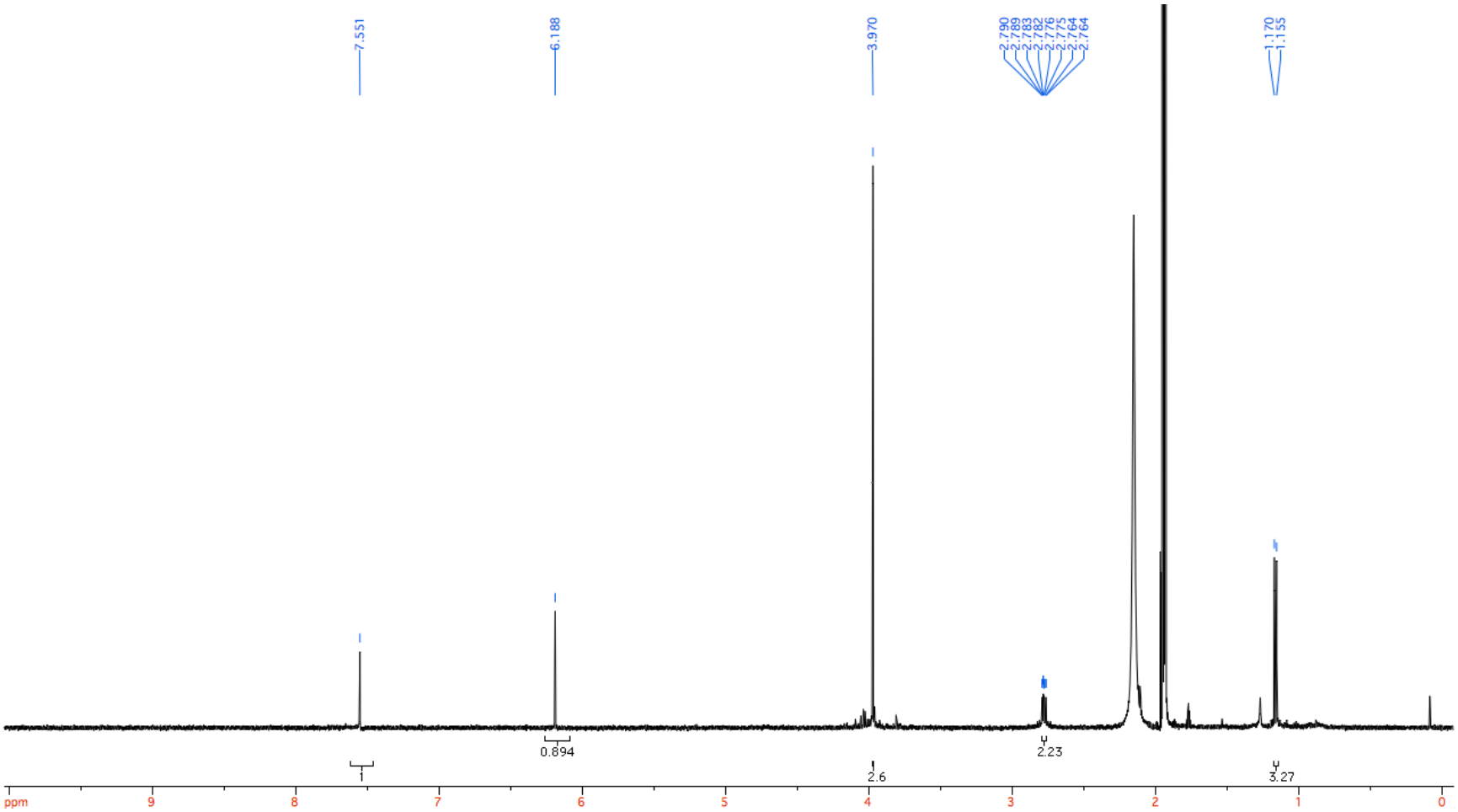


Figure S9. ^{1}H NMR (400 MHz, CD_3CN) of cercoquinone B (**10**) purified from *C. nicotianae* ΔCTB5.

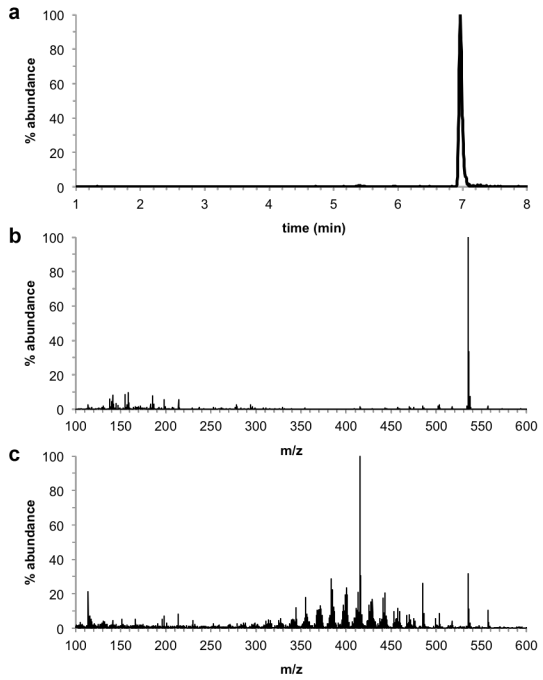


Figure S10. HRMS spectra for cercosporin (**1**). (a) Extracted ion chromatogram for *m*/*z* 535.1604, and (b) MS, (c) MS/MS for cercosporin.

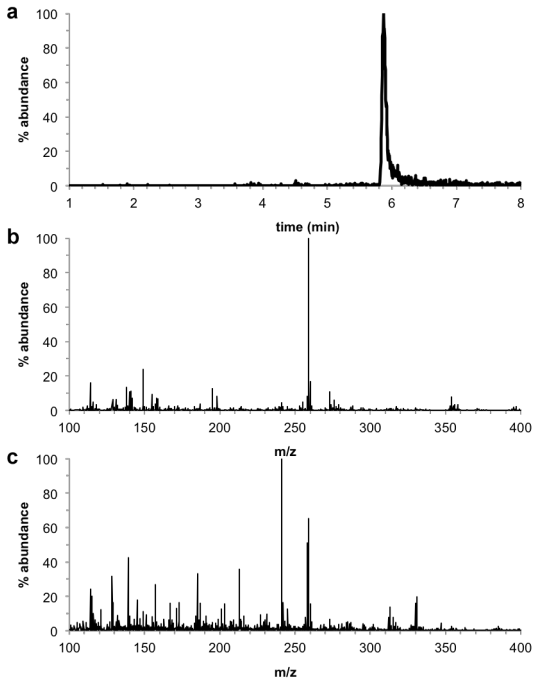


Figure S11. HRMS spectra for *nor*-toralactone (**6**). (a) Extracted ion chromatogram for *m*/*z* 259.0607, and (b) MS, (c) MS/MS for *nor*-toralactone.

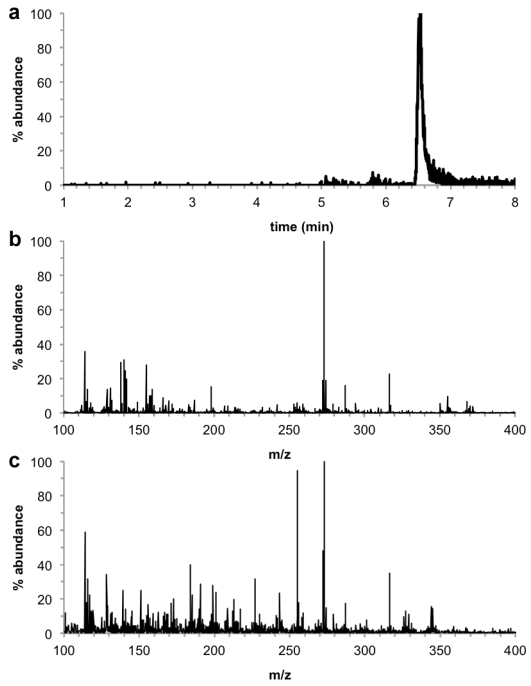


Figure S12. HRMS spectra for toralactone **(7**). (a) Extracted ion chromatogram for *m/z* 273.0763, and (b) MS, (c) MS/MS for toralactone.

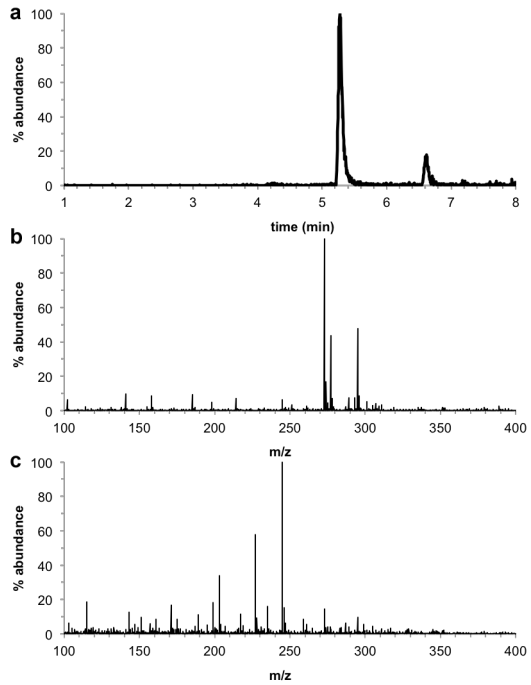


Figure S13. HRMS spectra for compound **8.** (a) Extracted ion chromatogram for *m/z* 273.0399, and (b) MS, (c) MS/MS for compound 8.

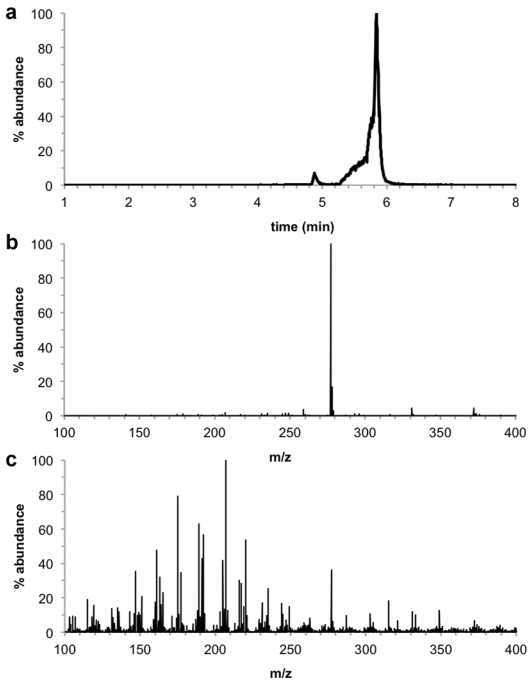


Figure S14. HRMS spectra for cercoquinone A **(9)**. (a) Extracted ion chromatogram for *m/z* 277.0712, and (b) MS, (c) MS/MS for cercoquinone A.

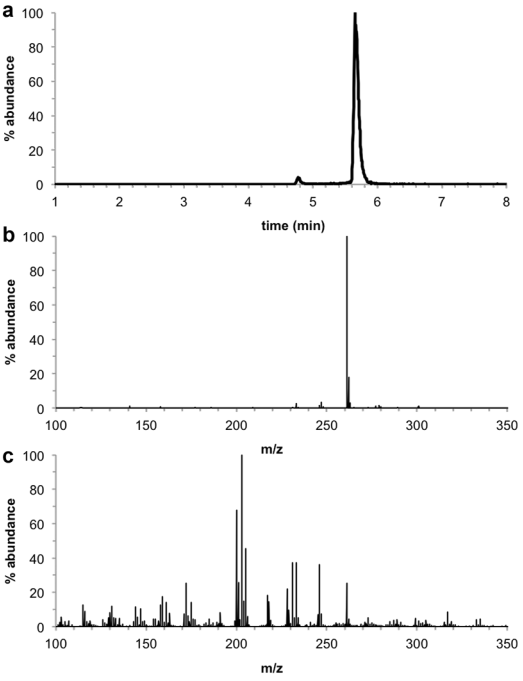


Figure S15. HRMS spectra for cercoquinone B **(10)**. (a) Extracted ion chromatogram for *m/z* 261.0763, and (b) MS, (c) MS/MS for cercoquinone B.

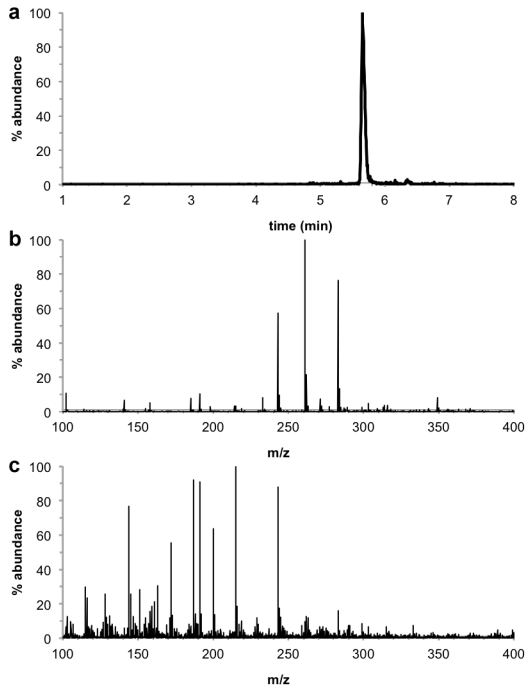


Figure S16. HRMS spectra for cercoquinone C (**12**). (a) Extracted ion chromatogram for *m/z* 261.0763, and (b) MS, (c) MS/MS for cercoquinone C.

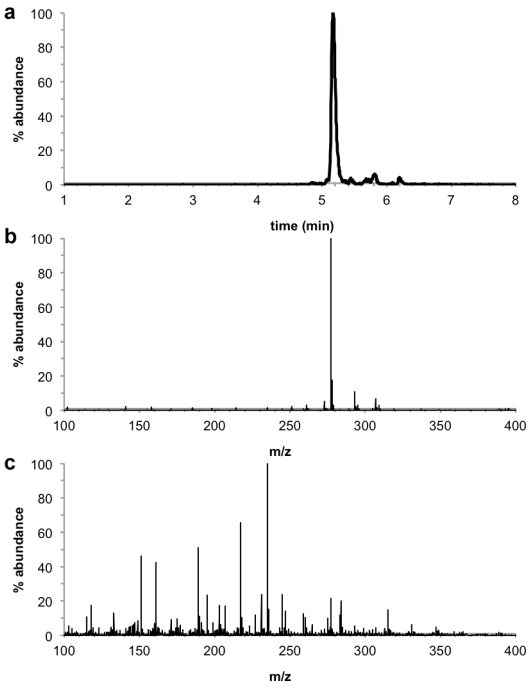


Figure S17. HRMS spectra for cercoquinone D (**11**). (a) Extracted ion chromatogram for *m/z* 277.0712, and (b) MS, (c) MS/MS for cercoquinone D.

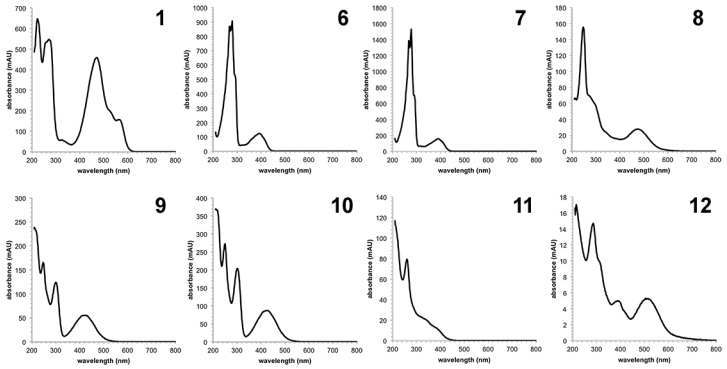
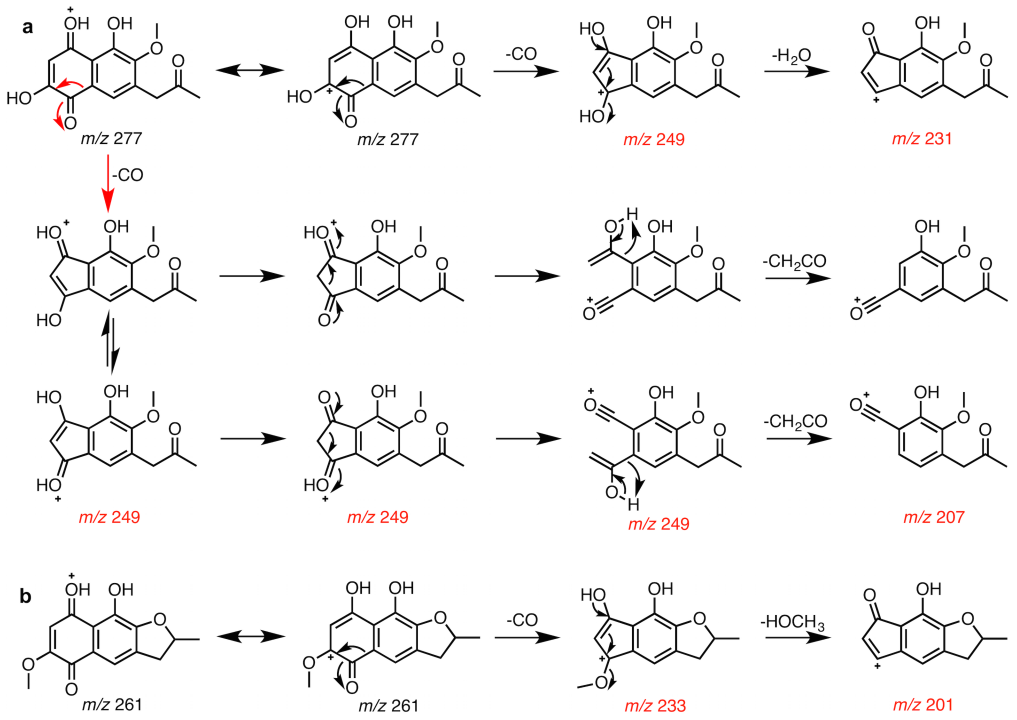


Figure S18. UV-visible spectra for cercosporin metabolites. The spectra for cercosporin (**1**),[12] *nor*-toralactone (**6**),[13] toralactone (**7**)[14] match those in the literature for these molecules.

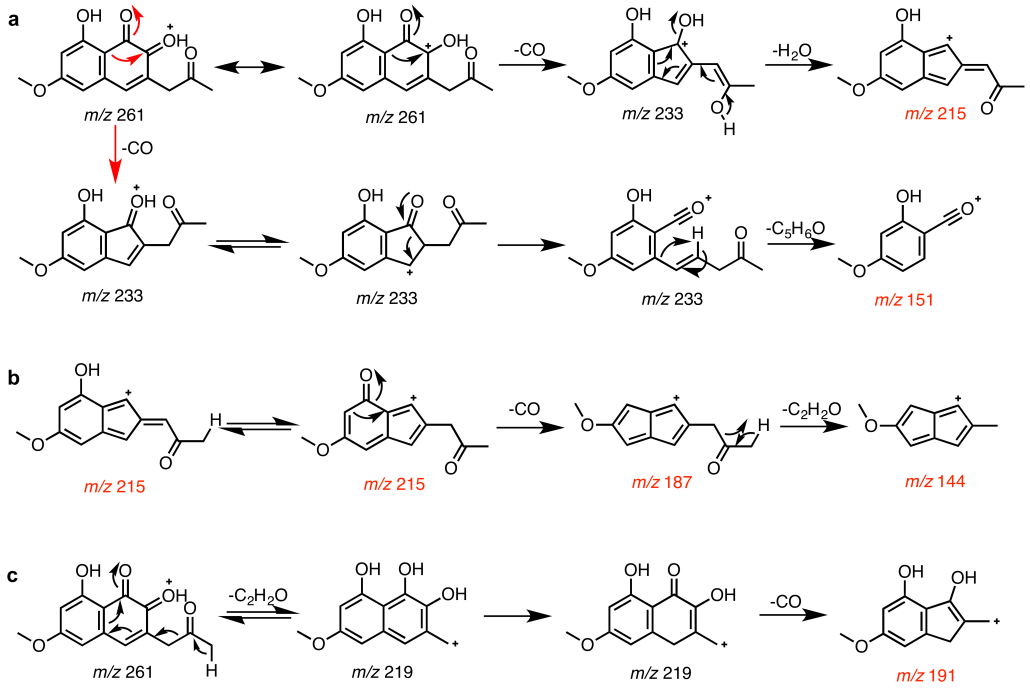
Mass Fragmentation of Cercoquinones A and B. Proposed structural assignments for new compounds cercoquinones A and B were supported by their ^{1}H NMR spectra, HRMS, mass fragmentation patterns, and UV spectra. The aromatic CH resonances in either compound are not coupled, supporting the 1,4-quinone oxidation pattern. Furthermore, each compound exhibited fragmentation ions diagnostic for 1,4-naphthoquinones. Mass fragmentation patterns for naphthoquinone congeners have been previously described.[15-17] Protonated 2-hydroxyl-1,4-naphthoquiones have been shown to fragment along two predominant pathways—ring contraction with elimination of CO and water resulting in a carbocation, or quinone ring-opening resulting in an oxonium ion. Fragment ions from both pathways were observed for cercoquinone A (*m/z* 207, 249, and 231, Scheme S1a), while fragmentations arising from only the first pathway were observed for cercoquinone B (*m/z* 233 and 201, Scheme S1b). The methoxyl substituent at the C2 position in cercoquinone B precluded the ring-opening mechanism. Thus, the ion fragments confirmed the sites of *O*-methylation in both cercoquinones A and B. Both cercoquinones A and B are similar to known compounds produced by various *Fusarium* species.[18,19]



Scheme S1. Mass fragmentation ions for cercoquinones A and B justify their structural assignment. Characteristic fragments of 1,4-napthoquinones are observed for (a) cercoquinone A and (b) cercoquinone B. Observed fragmentation ions are in red.

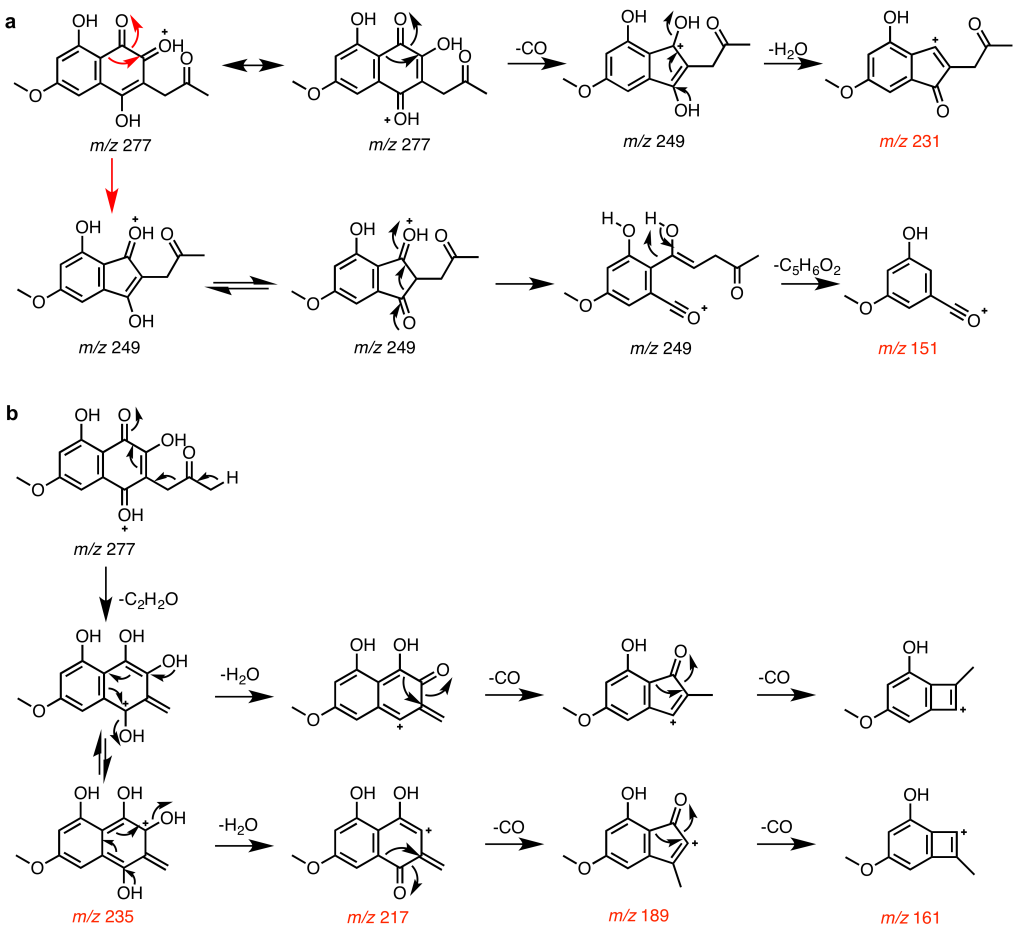
Mass Fragmentation of Cercoquinones C and D. Structural assignments of both cercoquinones C and D (**12** and **11**, respectively) were supported by characteristic mass fragments for 1,2-naphthoquinones and 1,4-naphthoquinones.[15-17] The cercoquinone C *m/z* 151 fragmentation ion corresponded to the product of the known fragmentation pattern for 1,2-napthoquinones (Scheme S2a). Interestingly, this fragmentation pathway established an intermediate that was primed for dehydration resulting in the *m/z* 215 ion (Scheme S2b). The *m/z* 215 ion underwent further fragmentation producing a pair of ions—*m/z* 187 and 144. The parent cercoquinone C ion was similarly primed for the elimination of the 2-oxopropyl substituent as ketene, yielding a resonance-stabilized benzylic cation (*m/z* 219, Scheme S2c). Although this ion was not observed, its ring-contracted CO-elimination ion was present (*m/z* 191). Additionally, a

predominant sodiated ion for cercoquinone C was observed in the HRMS—a common feature of 1,2-napthoquinones.[17]



Scheme S2. Mass fragmentation ions for cercoquinone C support its structural assignment. (a) Characteristic fragments of 1,2-napthoquinones are observed. (b) The 2-oxopropyl fragmentation ions derived from the *m/z* 215 ion. (c) The *m/z* 191 ion derived from the initial 2-oxopropyl fragment ion. Observed fragmentation ions are in red.

The cercoquinone D fragmentation pattern was characteristic for a 2-hydroxyl-1,4-naphthoquinone compound. The distinguishing ring-contracted ion (*m/z* 231) and the oxonium ion (*m/z* 151)—similar to those observed for cercoquinones A—were both evident (Scheme S3a). As with cercoquinone C, the 2-oxopropyl substituent was fragmented and eliminated as ketene with the participation of the quinone moiety (Scheme S3b). The resulting resonance-stabilized ion underwent a characteristic series of dehydrations and CO eliminations with ring-contractions generating a collection of distinctive ions (*m/z* 235, 217, 189, and 161).



Scheme S3. Mass fragmentation ions for cercoquinone D justify its structural assignment. (a) Characteristic fragments of 2-hydroxyl-1,4-napthoquinones are observed. (b) The fragmentation ions derived from the initial 2-oxopropyl fragment ion. Observed fragmentation ions are in red.

REFERENCES

(1) Choquer, M.; Dekkers, K. L.; Chen, H. Q.; Cao, L. H.; Ueng, P. P.; Daub, M. E.; Chung, K. R. *Mol. Plant Microbe Interact.* **2005**, *18*, 468-476.

(2) Chen, H. Q.; Lee, M. H.; Daub, M. E.; Chung, K. R. *Mol. Microbiol.* **2007**, *64*, 755-770.

(3) Dekkers, K. L.; You, B. J.; Gowda, V. S.; Liao, H. L.; Lee, M. H.; Bau, H. H.; Ueng, P. P.; Chung, K. R. *Fungal Genet. Biol.* **2007**, *44*, 444-454.

(4) Choquer, M.; Lee, M. H.; Bau, H. J.; Chung, K. R. *FEBS Lett.* **2007**, *581*, 489-494.

(5) Chen, H. Q.; Lee, M. H.; Chung, K. R. *Microbiology* **2007**, *153*, 2781-2790.

(6) Sambrook, J.; Russell, D. W. *Molecular cloning: A laboratory manual*; Cold Spring Harbor Laboratory Press, Plainview, NY, 2001.

(7) Udwary, D. W.; Merski, M.; Townsend, C. A. *J. Mol. Biol.* **2002**, *323*, 585-598.

(8) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.

(9) Korbie, D. J.; Mattick, J. S. *Nature Protocols* **2008**, *3*, 1452-1456.

(10) Voytas, D. In *Current Protocols in Molecular Biology*; John Wiley & Sons, Inc.: 2001.

(11) Aliverti, A.; Curti, B.; Vanoni, M. A. *Flavoprotein Protocols* **1999**, *131*, 9-23.

(12) Yamazaki, S.; Ogawa, T. *Agric. Biol. Chem.* **1972**, *36*, 1707-1718.

(13) Newman, A. G.; Vagstad, A. L.; Belecki, K.; Scheerer, J. R.; Townsend, C. A. *Chem. Commun.* **2012**, *48*, 11772-11774.

(14) Zhang, Z. J.; Yu, B. *J. Org. Chem.* **2003**, *68*, 6309-6313.

(15) Vessecchi, R.; Emery, F. S.; Galembeck, S. E.; Lopes, N. P. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 2101-2108.

(16) Vessecchi, R.; Emery, F. S.; Galembeck, S. E.; Lopes, N. P. *J. Mass Spectrom.* **2012**, *47*, 1648-1659.

(17) Vessecchi, R.; Emery, F. S.; Lopes, N. P.; Galembeck, S. E. *Rapid Commun. Mass Spectrom.* **2013**, *27*, 816-824.

(18) Tatum, J. H.; Baker, R. A.; Berry, R. E. *Phytochemistry* **1987**, *26*, 795-798.

(19) Studt, L.; Wiemann, P.; Kleigrewe, K.; Humpf, H. U.; Tudzynski, B. *Appl. Environ. Microbiol.* **2012**, *78*, 4468-4480.